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Suppression of Sebum Secretion with 13-*cis*-Retinoic Acid: Effect on Individual Skin Surface Lipids and Implications for Their Anatomic Origin*

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The contribution of the keratinizing epidermis to the human skin surface lipid film has been difficult to ascertain because, after its release from the epidermal cells, epidermally derived lipid inevitably becomes mixed with sebum. In the present study, the sustainable rates of production of the 5 neutral lipid classes found on the skin surface (triglycerides + free fatty acids, wax esters, cholesterol, cholesterol esters, and squalene) were measured on the foreheads of acne patients before, during, and following treatment with 13-*cis*-retinoic acid, a drug which suppresses sebum production profoundly. Since sebum production was high in the patients before treatment and was suppressed to a few percent of the pretreatment level in some of the patients during treatment, data covering a wide range of sebum production rates were obtained. By using squalene as a measure of sebum production and plotting the rates of production of the other lipid classes vs the rate of production of squalene, it was possible through extrapolation to estimate the residual (i.e., epidermal) rate of production of each lipid class at zero sebum production. The results indicated that epidermis releases triglycerides + free fatty acids and cholesterol to the skin surface. The cholesterol esters in freshly secreted skin surface lipids appeared to be almost entirely sebaceous in origin.

Measurements were also made of the percentages of cholesterol esters in lipid collected from the scalp after several days' accumulation and were compared to corresponding values for the forehead lipid. The percentages of cholesterol esters in scalp lipid tended to rise when sebum production was suppressed by the drug, rather than remaining relatively constant as occurred in the freshly secreted forehead lipid. This result indicated that epidermis may contribute to skin surface cholesterol esters, probably through skin surface esterification of epidermal cholesterol.

Human skin surface lipid consists of a mixture of sebum and of lipid derived from the keratinizing epidermis. Sebum is thought to be the sole source of the wax esters and squalene found in the surface lipid film [1,2]. However, the relative contributions of sebum and epidermal lipids to the skin surface pools of triglycerides, free fatty acids, cholesterol, and cholesterol esters have been difficult to define.

Epidermal components may be expected to form a larger proportion of total skin surface lipid on skin with low sebum production rates than on skin with high sebum production rates. Therefore, one approach to identifying epidermal lipids has been to measure the amounts of various skin surface lipid classes on skin at anatomic sites having differing levels of sebaceous gland activity. Using this approach, Greene et al, [2] concluded that the epidermis contributes triglycerides and/or free fatty acids, cholesterol, and cholesterol esters to the skin surface lipid film. Gray and Yardley [1] have shown, however, that human epidermis contains insignificant quantities of cholesterol esters. This discrepancy between the two studies in regard to the origin of cholesterol esters may result from a skin surface esterase acting to form cholesterol esters from secreted cholesterol.

In the present study, a wide range of variation in sebum production rates was induced in acne patients by treatment with oral 13-*cis*-retinoic acid (Accutane). The effect of this variation in sebum production on the rates of production of various skin surface lipids by forehead skin was studied, using a method involving absorption of freshly secreted lipid into bentonite clay and subsequent analysis of the collected lipid. From the results, it was possible to estimate the epidermal rate of production of various skin surface lipid classes. The possibility of skin surface esterification of cholesterol was studied by comparing the percentage of cholesterol esters in freshly secreted forehead lipid to that in lipid which had been allowed to accumulate on the scalp.

METHODS

Subjects and Treatment

The subjects were 18 acne patients (16 men and 2 women, ages 17–28 years), who were participating in a clinical trial of 13-*cis*-retinoic acid. Informed consent was obtained for all procedures. The patients received the drug for 20 weeks in dosages of 0.1, 0.5, or 1.0 mg/kg/day. Details of the effects of the drug on sebum production in these patients have been reported elsewhere [3].

Measurement of Skin Surface Lipid Production Rates

Skin surface lipid production rates were measured on 9 occasions in each patient: prior to treatment; at 2, 4, 8, 12, 16, and 20 weeks of treatment; and at 4 and 8 weeks after discontinuation of treatment. On each occasion, the collection sites on the patients' foreheads were first depleted of lipid by two sequential applications of a gel of bentonite clay in 10% aqueous ethanol containing 0.2% carboxymethylcellulose. A rectangular piece of nylon cloth was embedded in the clay as it was applied. When the cloth was later removed, the dry clay came off with it. The clay was left in place for a total of 14 h immediately prior to the beginning of the 3-h collection period. This preabsorption step is necessary in order to deplete accumulated sebum and to allow for the measurement of the sustainable rates of skin surface lipid production [4]. For the measurements, fresh bentonite clay was applied to the sites. During this application of the clay, 2 disks of Dacron mesh, 1.9 cm in diameter, were embedded in the clay about 1 cm apart on either side of the midline of the forehead. At the end of the 3-h collection period, the disks were removed, each bringing with it the clay from a 2.8-cm^2 area of skin.

After the disks were removed from the forehead, they were placed in separate vials containing 5 ml of ether. Several hours later, the ether extracts were transferred to screw-cap test tubes, the ether was evap-

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orated, and the lipid was redissolved in either 50 or 100 μl of toluene containing 2 mg/ml of a methyl oleate internal standard. Aliquots (4, 8, or 12 μl) of the toluene extracts were applied to 6 mm-wide lanes scored in thin-layer chromatography plates coated with 250-μm layers of silica gel G. Each sample was chromatographed on 2 separate plates. Plate 1 was developed in hexane and then in toluene. Plate 2 was developed in hexane, toluene, and twice to 10 cm in hexane:ether:acetic acid, 80:20:1. The developed plates were sprayed with 50% aqueous sulfuric acid and then charred by heating slowly to 220°C on an aluminum slab placed on a hotplate. After the plates had cooled, the charred spots were quantified by photodensitometry [5,6]. The amount of wax esters in each disk was calculated from plate 1 by dividing the densitometric peak area for the wax ester spot by the peak area for the methyl oleate spot and multiplying the result by the amount of methyl oleate (0.1 or 0.2 mg) added to the sample. An average of the right and left disks was calculated and converted to the traditional units of mg/10 cm^2/3 h (mg/disk/3 h ÷ 0.28 = mg/10 cm^2/3 h). The production rates of the other lipid classes were estimated by first calculating the percentage composition of each lipid extract from the peak areas on plate 2, using appropriate correction factors for carbon content [5]. The rate of production of each lipid class was then calculated using the relationship: rate of production of lipid X = (% lipid X/% wax esters) × rate of production of wax esters. Rates of production of triglycerides and free fatty acids were added together and these components treated as a single lipid class. This was done because skin surface bacteria can hydrolyze triglycerides and it is, therefore, not possible to know how much free fatty acid is released in free form and how much as triglycerides.

Analysis of Scalp Lipid

The patients had been instructed to shampoo only with Johnson's Baby Shampoo and to use no other hair products during the study. They refrained from shampooing for 1–2 days before the collection when they were off treatment, and for 1 week when they were on treatment. Scalp lipid was extracted by pouring 400 ml of 95% ethanol over the hair and scalp of the patients. (Immediately following this procedure the patients rinsed their hair thoroughly with water.) The composition of the scalp lipid was determined by thin-layer chromatography and photodensitometry, as described above for the forehead disk extracts.

RESULTS

Contribution of the Epidermis to Skin Surface Lipid

By plotting the production rate of each lipid class vs the production rate of squalene and extrapolating to zero squalene production, an estimate of the amount of each lipid class that was derived from the epidermis could be obtained. Figs 1 and 2 show the results of plotting all of the points from all of the patients, regardless of the dose level of 13-*cis*-retinoic acid that they received. The regression lines both for triglycerides + free fatty acids vs squalene and for cholesterol vs squalene have positive intercepts on the y axis (Figs 1*A*, 2*A*). The y intercepts provide estimates of the amount of triglycerides + free fatty acids and of cholesterol which are not derived from sebum and, therefore, presumably arise from epidermis. In contrast, the production rates of wax esters (Fig 1*B*) approach zero as squalene production approaches zero. (The relationship between wax esters and squalene is not linear and the regression line does not pass through the origin. However, it can be seen from the data points that both components tend toward zero together.) Fig 2*B* shows that the amount of cholesterol esters contributed by epidermis is probably very small.

Fig 3 shows the values obtained for each individual patient's production rates of triglycerides + free fatty acids, cholesterol, and cholesterol esters. The values were obtained by calculating regression lines as in Figs 1 and 2, except that the regression lines were calculated separately for each patient using the data from the 9 collections made before, during, and following treatment. The points are grouped according to the dose of 13-*cis*-retinoic acid received. The dose of the drug appeared to have no effect on the average values for the epidermal production rates of the various lipid classes. The calculated epidermal production rates of triglycerides + free fatty acids are more variable among individuals than are the rates for cholesterol and cholesterol esters. The average epidermal production rate of cholesterol esters from the regression lines for the 18 patients

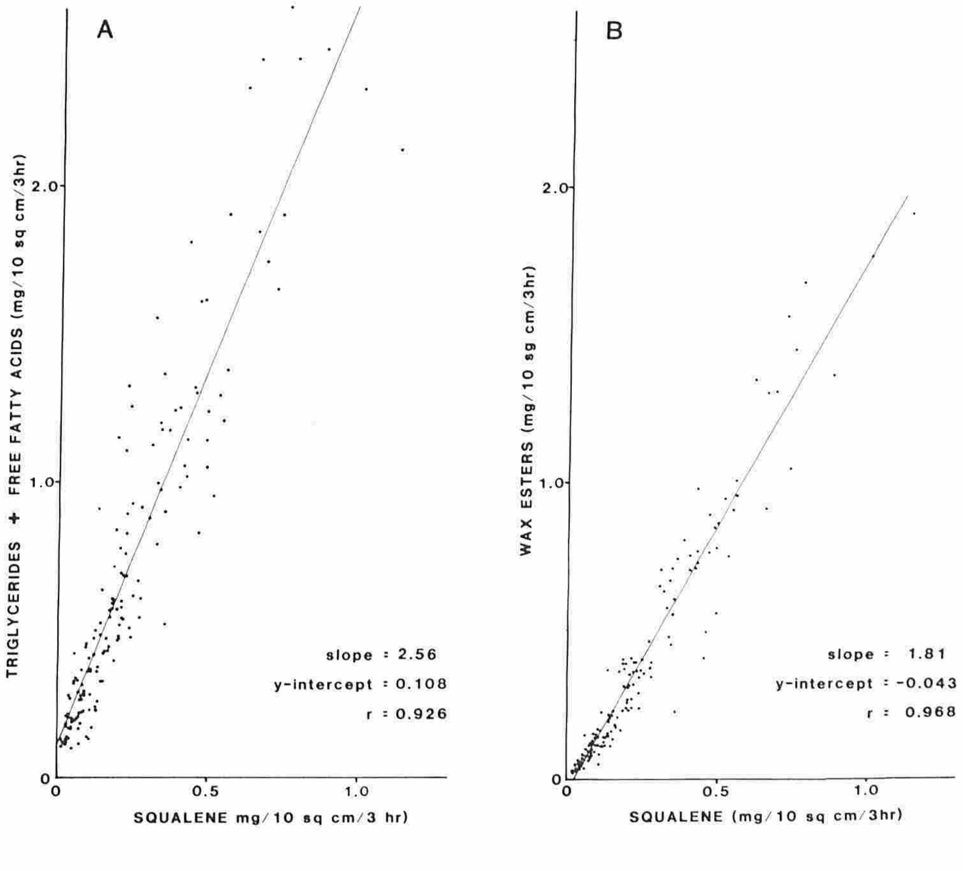

FIG 1. Plots of the amounts of triglycerides + free fatty acids (*A*) and wax esters (*B*) vs amounts of squalene produced on 10 cm^2 of forehead skin in 3 h. Skin surface lipid was collected from 18 acne patients who were sampled 9 times: once before, 6 times during, and twice following oral treatment with 13-*cis*-retinoic acid.

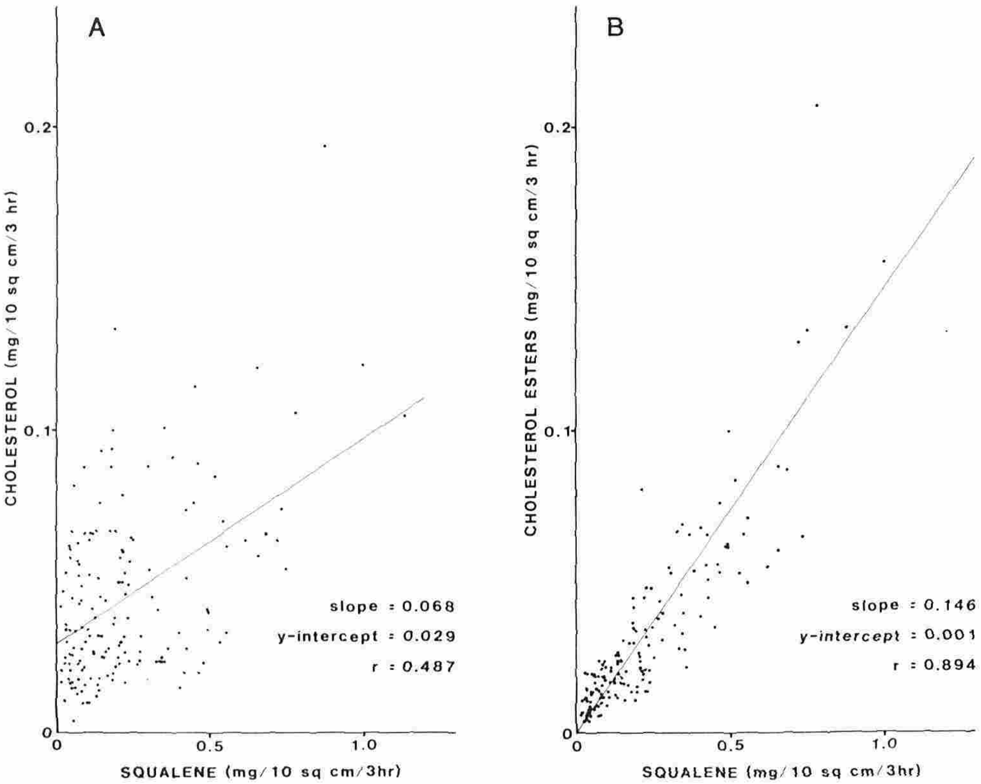

FIG 2. Plots of the amounts of cholesterol (*A*) and cholesterol esters (*B*) vs amounts of squalene produced on 10 cm^2 of forehead skin in 3 h. Data were obtained from the same samples as in Fig 1.

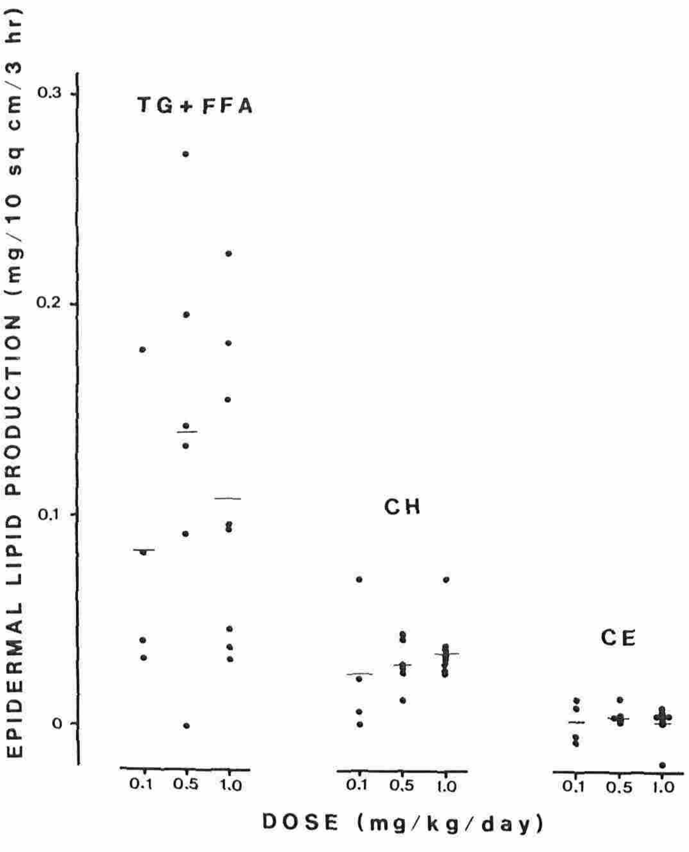

FIG 3. Estimated rates of production of 3 neutral lipid classes on the foreheads of 18 acne patients treated with different doses of 13-*cis*-retinoic acid. Each point represents the y intercept of a regression line from a plot similar to those in Figs 1 and 2, except that each plot included the values from only 1 patient.

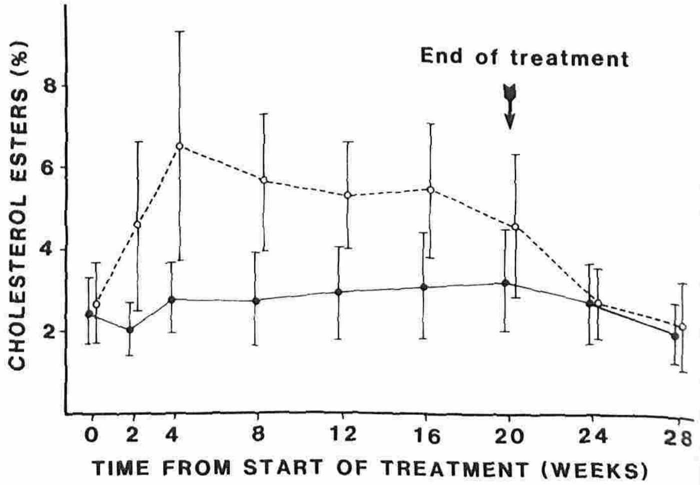

FIG 4. Percentages of cholesterol esters in lipid recovered from the forehead (*solid circles*) and scalp (*open circles*) of 18 acne patients before, during, and following treatment with 13-*cis*-retinoic acid. The points show means ± SD. The difference between forehead and scalp was significant at the 0.01 level for all points from week 2 through week 20 (paired *t*-tests). The decline in the percentage of cholesterol esters in the scalp lipid between 4 and 20 weeks was also significant ($r = -0.905$, $p < 0.05$).

calculated separately was 0.004 mg/10 cm^2/3 h, in contrast to 0.001 mg/10 cm^2/3 h from the regression line in Fig 2*B*. However, if the regression line in Fig 2*B* was recalculated omitting the two highest (and very deviant) points, a value of 0.004 mg/10 cm^2/3 h was obtained.

Percentage of Cholesterol Esters in Forehead and Scalp Lipid

In Fig 4, it can be seen that the percentage of cholesterol esters in the forehead lipid remained relatively constant during drug treatment. However, in the scalp lipid the percentage of cholesterol esters more than doubled after 4 weeks on treatment. The difference between forehead and scalp values was significant for all collections made during treatment. There was a significant decline between 4 and 20 weeks in the percentage of cholesterol esters in scalp lipid, but values did not return to pretreatment levels until treatment ended.

DISCUSSION

The Origin of Squalene and Wax Esters

Squalene and wax esters are regarded as unique products of the sebaceous glands, since neither is found in isolated epidermal cells, except in trace amounts which may represent contamination by sebum [1]. Moreover, both these components tend to be low on the skin of young children [7] in whom sebaceous glands are small because of low levels of circulating androgens [8]. In unpublished results, we have found that in some 6- to 8-year-old children squalene production on the forehead is undetectable and the production of wax esters is very low (less than 0.005 mg/10 cm^2/3 h).

Effect of 13-cis-Retinoic Acid on Wax Ester Production

It has been observed previously that the wax ester/squalene ratio is decreased in the skin surface lipid of subjects being treated with 13-*cis*-retinoic acid [9,10]. Commenting on this effect, Strauss et al [9] postulated that squalene synthesis may be preferentially preserved when sebum secretion is suppressed by the drug or, alternatively, epidermal lipid may contain some squalene. The results of the present study indicate that the wax ester/squalene ratio falls because wax ester synthesis is suppressed more than that of other sebaceous lipids. Linear relationships were observed between the production of squalene and the production of lipids other than wax esters (Figs 1, 2), while when the rates of production of the various lipid classes were plotted vs the rate of production of wax esters (not shown) nonlinear relationships were observed. Therefore, it must be the wax esters that change relative to other sebaceous lipid classes. The reason for this change remains to be determined. The observation that rates of wax ester and squalene production tend toward zero together, although in a nonlinear manner (Fig 1*B*), is in agreement with the evidence mentioned above that skin surface squalene is entirely sebaceous in origin.

The Origins of Triglycerides + Free Fatty Acids and Cholesterol

The results of the present study, indicating that triglycerides + free fatty acids and cholesterol are released to the skin by the epidermis, are in agreement with the reports of Nicolaides [11] and of Gray and Yardley [1] that epidermis contains these lipid classes. Gray and Yardley found that triglycerides, free fatty acids, and cholesterol each accounted for 25% of the neutral lipids of human epidermal cells. Our data indicate a somewhat lower proportion of cholesterol relative to triglycerides + free fatty acids. One possible explanation of this discrepancy is that, in some patients, 13-*cis*-retinoic acid increased the amount of triglycerides + free fatty acids released by the epidermis. (See next section).

On areas with any substantial rate of sebum secretion, sebaceous glands would be quantitatively more important than epidermis as the source of skin surface triglycerides + free fatty acids, since these components together make up about 55% of sebum [2]. The role of the sebaceous glands in supplying skin surface cholesterol is not entirely clear. Isolated sebaceous glands contain some cholesterol [12], as would be expected considering the importance of cholesterol in membrane structure. Before sebum is secreted, however, cellular membranes are broken down and much of the cholesterol may become esterified with fatty acids released from phospholipids. In Fig 2*A* the rate of cholesterol secretion can be seen to increase significantly with the rate of sebum secretion. However, when regression lines were calculated for each patient separately, the *r* values (not shown) for 9 of them indicated no significant increase of cholesterol secretion with increased squalene secretion. This suggests that there may be individual variation in the extent of cholesterol esterification in terminally differentiating sebaceous cells.

Effect of 13-cis-Retinoic Acid on Epidermal Lipid Production

The possibility exists that rates of epidermal lipid production are influenced by 13-*cis*-retinoic acid. Indeed, Plewig, Nikolowski, and Wolff [13] reported that, in some patients on 13-*cis*-retinoic acid, the corneocytes lining the follicular wall accumulate abnormal lipid droplets. Since our study depended on the sebum-suppressing effect of 13-*cis*-retinoic acid, there was no way to compare epidermal lipid production in patients and untreated control subjects. However, it was possible to compare patients on the three different dose levels. The results (Fig 3) indicated that there was no effect of dose size at the levels used. However, there was considerable variability among individual patients on all three doses in the estimated epidermal production rate of triglycerides + free fatty acids. Possibly this variability results from different effects of the drug on different individuals.

The Origin of Cholesterol Esters

The present study supports other evidence that sebaceous glands produce cholesterol esters. Thus, although Kellum [14] did not detect cholesterol esters in lipid from isolated sebaceous glands, a later study using a more sensitive method of detecting lipids on thin-layer chromatograms indicated that lipid from isolated sebaceous glands contains about 2% cholesterol esters [12]. In a study of in vivo sterol secretion, Nikkari, Schreibman, and Ahrens [15] found that lipid collected from the forehead, an area of high sebum secretion, had more cholesterol esters relative to free cholesterol than did lipid from the feet, which have few sebaceous glands. A sebaceous origin for most skin surface cholesterol esters can also be inferred from the report by Gray and Yardley [1] that human epidermal neutral lipid contains only about 2% cholesterol esters, an amount which is too little to account for a 2.5% concentration in skin surface lipid. Finally, the fact that skin surface cholesterol esters contain types of fatty acyl chains thought to be unique to sebum (e.g., various methyl-branched and Δ6-unsaturated chains [16]) is also evidence for a sebaceous origin of at least the fatty acid moieties.

Whether the epidermis releases any cholesterol esters to the skin surface remains in doubt. Our results shown in Fig 4 indicate that a small amount of cholesterol esters may be derived from the epidermis, but it was not possible to distinguish direct epidermal secretion from skin surface esterification (next section).

Role of Skin Surface Esterification

In skin surface lipid from areas of high sebum production the percentage of cholesterol esters is about 2.5%. Any change in this percentage when sebum production is drastically reduced should give an indication of the role of the epidermis in the production of skin surface cholesterol esters. There are three possibilities: (1) If the percentage of cholesterol esters is about 2.5 in both sebum and in the neutral epidermal lipids, reducing sebum production should have no effect on the percentage of cholesterol esters in skin surface lipid. (2) If there are no cholesterol esters contributed by epidermis, reducing sebum production should reduce the percentage of cholesterol esters in skin surface lipid because, with a small amount of sebum, dilution of its components with epidermal lipid would become noticeable. (3) If the nonpolar lipids of epidermis contain considerably more than 2.5% cholesterol esters, then an increase in the percentage of cholesterol esters in skin surface lipid should occur when sebum production is reduced.

These various possibilities were tested and the results are shown in Fig 4, which compares the percentage of cholesterol esters in forehead lipid and scalp lipid. It can be seen that reduction of sebum production caused little change in the percentage of cholesterol esters in forehead lipid, suggesting that there is an equal percentage of cholesterol esters in both sebum and epidermal lipid. However, in scalp lipid from the same patients there was an increase in the percentage of cholesterol esters, suggesting that scalp epidermal lipid may contain much more than 2.5% cholesterol esters. While it is not impossible that scalp epidermal lipid has a different composition than forehead lipid, a more likely explanation for the discrepancy between the two sites is skin surface esterification of epidermal cholesterol on the scalp. The forehead lipid was collected after a long preliminary depletion step and the samples, therefore, contained freshly secreted lipid. The scalp lipid, in contrast, was collected after several days' accumulation on the scalp, allowing time for chemical transformation to occur.

Rates of sebum secretion were not measured on the scalp, but by assuming that they were about the same as rates measured on the forehead at the times that the scalp lipid was collected, some calculations can be made. The average pretreatment rate of sebum production on the forehead was 3.3 mg/10 cm^2/3 h. During the on-treatment period from 4 through 20 weeks, average lipid production on the forehead was 0.6 mg/10 cm^2/3 h. To increase the cholesterol ester content of this lipid from 2.5% to 6% would require an input of cholesterol esters from the epidermis at the rate of 0.021 mg/10 cm^2/3 h. Allowing for a 66% increase in molecular weight in converting cholesterol to cholesterol esters, 0.021 mg of cholesterol esters could be provided by the esterification of 0.013 mg of cholesterol. From Fig 2*A*, the epidermal rate of cholesterol production on the forehead was about 0.029 mg/10 cm^2/3 h. Assuming this is the rate of production on the scalp, then there was about 50% esterification of scalp epidermal cholesterol in the average patient.

The skin-dwelling organism *Propionibacterium acnes* has been shown to be able to esterify cholesterol in vitro [17] and seems a likely candidate for the skin surface conversion. The gradual decline in the percentage of cholesterol esters in scalp lipid during treatment tends to implicate *P. acnes* rather than a keratinocyte esterase [16], since the population of *P. acnes* has been shown to decline during treatment with 13-*cis*-retinoic acid [10].

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